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PRINCIPAL INVESTIGATOR: Stephen R. Hann, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203-6869

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**6. AUTHOR(S)**
Stephen R. Hann, Ph.D.

E-Mail: steve.hann@vanderbilt.edu

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**14. ABSTRACT**
Since many breast tumors have deregulated c-Myc, we hypothesize that an ARF mimic would be a valuable therapeutic agent for breast cancer to inhibit c-Myc-induced transformation/tumorigenesis without inhibiting normal cell proliferation or apoptosis. To test this hypothesize we will generate the reagents and establish the assays for the high throughput screening of chemical compounds that mimic ARF and analyze the candidate chemical compounds identified by the screen in molecular and biological assays. If successful, these studies would directly lead to a therapeutic agent to inhibit breast tumors that are known to harbor deregulated/elevated c-Myc.

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## Introduction

Numerous studies have demonstrated an essential role for the c-*myc* gene in the control of normal cell cycle progression. Deregulated c-*myc* expression has been demonstrated in many types of human cancer, including breast cancer. Elevated/deregulated c-Myc has been shown to induce hyperproliferation, induce apoptosis in cells deprived of survival factors, transform cells in vitro, and cause tumorigenesis. The c-Myc protein is a transcription factor that has been shown to both upregulate and downregulate a variety of target genes. Recently, we have found that the tumor suppressor, $p19^{ARF}$ (ARF) binds to c-Myc and differentially controls c-Myc transcriptional and biological functions. It has been previously shown that ARF is induced by c-Myc and mediates p53 activation. It has also been shown that inactivation of the ARF-Mdm2-p53 pathway allows oncogenic c-Myc to drive cell cycle progression without apoptosis. We have found that ARF binding to c-Myc protein inhibits c-Myc transactivation and c-Myc-induced hyperproliferation and transformation without affecting normal proliferation. Furthermore, ARF does not inhibit repression of c-Myc target genes and actually appears necessary for Inr-mediated repression and enhances c-Myc-induced apoptosis.

## Body

It has been shown that deactivation of c-Myc results in the sustained regression of tumors, suggesting that c-Myc is an excellent target for the inhibition of tumorigenesis. It has also been demonstrated that specific chemical compounds can antagonize Myc/Max dimerization and inhibit transformation. However, inhibition of the c-Myc/Max heterodimerization is not specific, since Max is critical for all functions of c-Myc and would likely inhibit normal cell proliferation and apoptosis. The inhibition of c-Myc-induced transformation by ARF is highly specific, since ARF does not inhibit c-Myc normal cell proliferation or apoptosis induced by c-Myc. Since many breast tumors have deregulated c-Myc, we hypothesize that an ARF mimic would be a valuable therapeutic agent for breast cancer to inhibit c-Myc-induced transformation/tumorigenesis without inhibiting normal cell proliferation or apoptosis. Before we could accomplish our goals of generating the reagents and establish the assays for the high throughput screening of chemical compounds that mimic ARF and analyzing the candidate chemical compounds identified by the screen in molecular and biological assays, we first needed to establish that a small ARF molecular mimic could affect tumor cell proliferation.

## Key Research Accomplishments

As proof of principle that ARF mimics could inhibit c-Myc activity we used a small ARF peptide. This peptide (amino acids 26-44) was synthesized and used to treat DKO MEFs (Arf/p53 double null). There was no effect on cells with inactive c-Myc, but c-Myc-induced hyperproliferation was inhibited by the peptide. We have also used this peptide to treat breast cancer cells. The proliferation of metastatic, fast-growing breast and colon cancer cells was inhibited in a dose-dependent fashion. In contrast, the proliferation of non-metastatic slow-growing breast cancer cells was not affected by the ARF peptide. These studies and these cells provide the rationale and starting material for the screening for chemical mimics. Chemical screening was initiated, but due to the limited budget and time of this award and the prohibitive cost of continuing thses experiments we could not finish these experiments.

## Reportable Outcomes

**c-Myc colocalizes with a small ARF fragment.** ARF is normally localized to the nucleolus. Upon c-Myc overexpression, we found that ARF is relocalized to the nucleoplasm where it colocalizes with c-Myc. These results suggest that c-Myc interacts with endogenous ARF in the nucleoplasm and prevents its nucleolar translocation. However, ARF can also influence c-Myc localization when ARF is exogenously expressed in some types of cells. As shown in Figure 1A-C, high overexpression of ARF-CFP in Cos cells results in localization of c-Myc-YFP to nucleoli. As an initial experiment to identify the region of ARF that interacts with c-Myc, we examined a peptide of ARF containing a region that has been previously shown to interact with Foxm1b and Mdm2 and mediate nucleolar localization. To determine whether this ARF peptide could alter the localization of c-Myc, we performed fluorescence

microscopy on Cos cells transiently expressing a chimeric protein of the ARF sequence (amino acids 26-44) and GFP. As shown in Figure 1D-E, expression of GFP-ARF 26-44 caused c-Myc to localize to the nucleoli as efficiently as full length ARF protein. We have also confirmed by co-immunoprecipitation experiments that this GFP-ARF 26-44 interacts with c-Myc (data not shown).

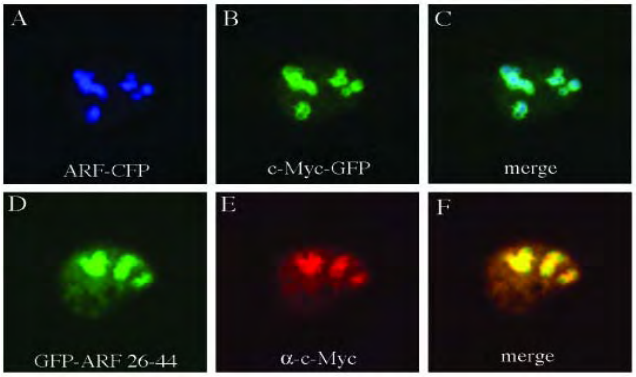


**Fig 1. Amino acids 26-44 of ARF are sufficient to target c-Myc to nucleoli.** Cos-7 cells were cotransfected with ARF-CFP and c-Myc-GFP (A-C) or with GFP-ARF 26-44 and untagged c-Myc (D-F). Forty-eight hours after transfection, cells were fixed using paraformaldehyde and, for D-F, cells were immunostained using anti-c-Myc. Cells were visualized with fluorescence microsopy using a 63X objective and the appropriate filters.

We then synthesized an ARF26-44 peptide with 3 arginines on the N-terminus to enhance uptake, which we termed RN22. Addition of basic amino acids has been demonstrated to allow the delivery of biologically active proteins into mouse cells and tissues. Typically, either 9 arginines or the Tat48-57 basic peptide is added to enhance uptake, but since the ARF26-44 peptide already has 6 basic amino acids we only added 3 more arginines. After confirming that the RN22 peptide was taken up by Cos cells and was found in the nucleus and nucleoli (data not shown), we examined the effect of RN22 on cellular proliferation. We have previously shown that ARF overexpression inhibited c-Myc-induced hyperproliferation in Rat1a fibroblasts. To induce c-Myc activity we use c-Myc fused to a modified estrogen receptor (ER). The activity of c-MycER is induced by hydroxytamoxifen (OHT). To examine the effects of RN22 we treated ARF null/p53 null (DKO) mouse embryo fibroblasts expressing c-MycER. Figure 2 demonstrates that there is a dose-dependent inhibition of cellular proliferation when c-MycER was activated by OHT and treated with increasing amounts of RN22, but there was no effect when an unrelated control peptide (PD22) was added. In addition, there was no significant effect of RN22 on DKO MEF proliferation without OHT activation (data not shown).

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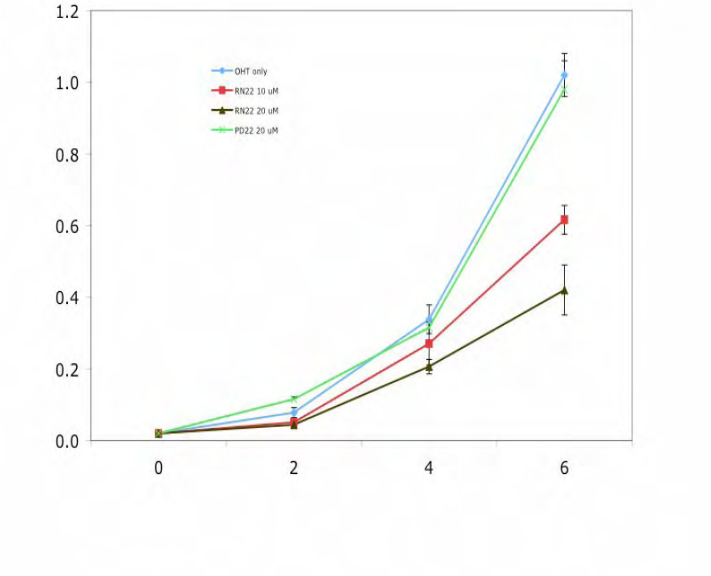


**Figure 2. RN22 peptide inhibits c-Myc-induced hyperproliferation of DKO MEFs.** p53-/- ARF-/- (DKO) MEFs stably expressing c-MycER were plated at a density of $2.5x10^4$/35 mm well. After 24 hours, the cells were left untreated or were treated with OHT to activate c-MycER. The cells were also treated with the RN22 ARF or the PD22 control peptide at the indicated concentration or with DMSO (vehicle). The quantity of living cells was determined every 2 days using the colorimetric MTT assay measuring the absorbance at 590 nm.

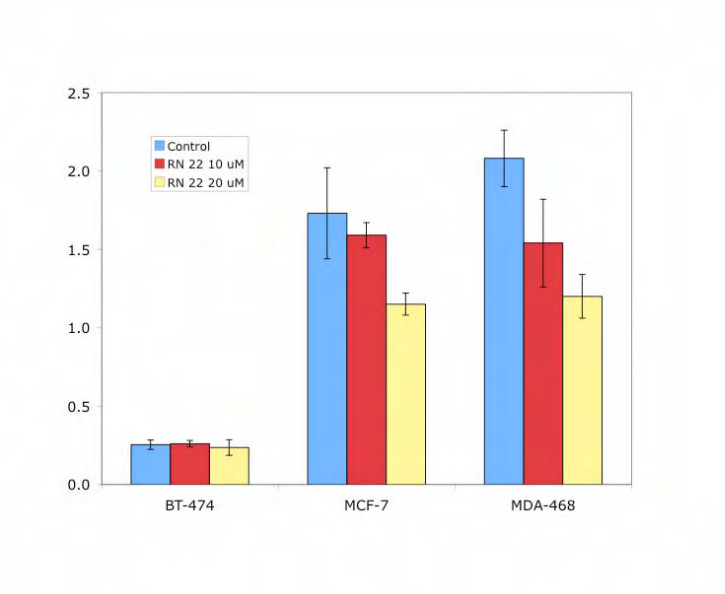


**Figure 3. RN22 peptide inhibits proliferation of breast cancer cell lines.** BT-474, MCF-7, or MDA-468 cells were treated with the RN22 peptide at the indicated concentration or with DMSO (vehicle) and the quantity of living cells was determined after 6 days.

Therefore, the ARF RN22 peptide specifically inhibits c-Myc-induced hyperproliferation, without affecting normal proliferation. Since breast cancer cell lines have been shown to have elevated c-Myc, we treated several different human breast cancer cell lines with RN22. Figure 3 shows that RN22 did not significantly effect the proliferation of the slow-growing BT-474 cells, whereas there was a dose-dependent inhibition of proliferation of the fast-growing MCF-7 and MDA-468 cells.

**Summary and Significance of Our Studies.** We have found that ARF binds c-Myc directly and that they colocalize in the nucleoplasm. ARF binding to c-Myc protein inhibits c-Myc-induced hyperproliferation of p53 null MEFs and Rat1a cells and soft agar growth of Rat1a cells, while enhancing c-Myc-induced apoptosis. Significantly, high levels of ARF do not affect normal cell proliferation. Our data show that a small 19 amino acid ARF fragment fused with GFP can bind and colocalize with c-Myc. We also found that an ARF peptide, RN22, inhibits c-MycER-induced hyperproliferation and inhibits the proliferation of, fast-growing breast cancer cell lines, without an apparent effect on slow-growing cells. This suggests that ARF peptides will also be able to inhibit the transforming activity of c-Myc, either by inhibiting proliferation or enhancing apoptosis. Considering that many metastatic breast cancer cell lines have high levels of c-Myc and have lost expression of ARF, breast cancer cell proliferation and metastasis may be inhibited by treatment with ARF peptides and ultimately chemical mimics.